The role of cutaneous Aβ fibers in human nocifensive functions: Nerve block study on painful withdrawal reflex responses

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Running title: Role of Aβ fibers in painful withdrawal reflex responses

Abstract

The nociceptive withdrawal reflex (NWR) is a protective limb withdrawal response triggered by noxious stimuli, used to assess spinal nociceptive excitability. Conventionally, the NWR is understood as having two reflex responses: a quick (short-latency) response mediated by Aβ fibers, considered tactile, and a delayed (long-latency) response mediated by Aδ fibers, considered nociceptive. Yet, some studies challenge this distinction, suggesting a nociceptive component in the quick response. Further, high-threshold mechanoreceptors with conduction velocities similar to Aβ tactile afferents have been identified in human skin. In this study, we investigated the contribution of Aβ-fiber inputs to pain perception and NWR signaling using intradermal electrical stimulation before, during, and following recovery from a preferential Aβ-fiber ischemic block. We recorded a total of 198 NWR responses. No dual reflex responses occurred. The current intensity required to evoke the NWR was manifold higher than the perceptual pain threshold, indicating that NWR did not occur before pain was felt. During the preferential Aβ-fiber block, neither pain nor reflex could be evoked at the pre-block thresholds. Although delayed pain could be evoked at higher stimulus intensities, the reflex could not be evoked despite a two-fold increase in the pre-block current intensity and a five-fold increase in the pre-block pulse duration. Our findings underscore the importance of very fast-conducting cutaneous afferents in pain perception and NWR signaling during intradermal electrical stimulation. While pain perception involves multiple afferent classes, the absence of NWR during the block suggests an important contribution of cutaneous Aβ inputs in driving our nocifensive behaviors.

Background

Pain is complex, involving cognitive, genetic, and psychosocial factors (Fillingim, 2017; Khera & Rangasamy, 2021; Rischer et al., 2020; Willer, 1980). Currently, pain assessment mainly relies on individuals' self-report which has limitations. In the early 20th century, reflexes were extensively studied by Sherrington who observed coordinated muscle movements, such as flexion followed by stepping movements, that correlated with the intensity of noxious stimulation (Sherrington, 1910). This phenomenon is often termed the nociceptive withdrawal reflex (NWR) and involves a complex interplay between top-down and bottom-up influences (Andersen, 2007; Carstens et al., 1990; Rhudy et al., 2005; Sandrini et al., 2005). It was initially suggested that the NWR may serve as an objective readout of pain, but several studies have since highlighted that the relationship between pain and reflex is not clearcut (de Willer, 1977; Dowman, 1991; Ellrich & Treede, 1998; Hugon, 1973; Popescu et al., 2010; Ruscheweyh et al., 2011; Ydrefors et al., 2020). Nonetheless, the reflex is a useful tool for monitoring the excitability of nociceptive spinal systems.

The NWR is often reported as consisting of two distinct electromyographic (EMG) responses with a silent period (Grimby, 1963; Hagbarth, 1960; Hugon, 1973). These responses, RII and RIII, are attributed to different peripheral afferents: RII to large, thickly myelinated afferents with Aβ-fiber conduction velocities (CV) and RIII to small, thinly myelinated afferents corresponding to Aδ-fiber CVs (Lloyd, 1943). It is generally believed that the RII is non-painful, and it is the RIII that represents spinal nociceptive signaling (Danziger et al., 1998; Hugon, 1973; Shahani & Young, 1971). Thus, it is common practice to exclude the first NWR component from the reflex analysis. However, there is no consensus on where in time the separation between RII and RIII should occur. Using a 90-ms cutoff, for instance, we found that NWR responses shorter than 90 ms were just as painful as those that were ≥90 ms (Thorell et al., 2023). Further, the NWR may comprise of single EMG responses occurring at varying latencies and independent of stimulation intensity, questioning the involvement of different peripheral afferents (Shahani & Young, 1971; Thorell et al., 2023).

We recently reported that human skin is equipped with a specific class of high-threshold mechanoreceptors with Aβ conduction velocities. These receptors encode noxious mechanical stimuli and produce painful percepts when selectively activated through low-current intraneural stimulation (Nagi et al., 2019; Yu et al., 2023). This discovery raises the question of whether Aβ inputs contribute

to painful NWR signaling in humans. Part of the ambiguity in the literature around latencies is due to the paucity of direct recordings from Aδ afferent fibers in humans, thereby relying on indirect measurements to infer their conduction velocities. Indeed, in animals, the conduction velocity of D-hair afferents is used as a cutoff between Aβ and Aδ populations (Djouhri & Lawson, 2004); however, D-hair afferents have not yet been characterized in humans. To work around this problem, we employed preferential Aβ-fiber conduction blocks and tested pain and reflex responses evoked by intradermal electrical stimulation before, during, and upon recovery from the block. Nerve conduction blocks are widely used in the somatosensory field to study the functions of primary afferent fibers (Mackenzie et al., 1975; Nagi et al., 2011; Torebjork & Hallin, 1973). In this study, we found that pain and reflex at pre-block thresholds were abolished during the block. Although delayed pain could be evoked at higher stimulus intensities, the reflex could not be evoked during the block despite considerable increases to the pre-block stimulus intensities. This suggests an important contribution of Aβ-fiber inputs to human pain perception and reflex signaling.

Methods

Participants

Twenty-five healthy participants (17 males and 8 females), aged 18-39 years, took part in this study. The exclusion criteria included neurological and musculoskeletal disorders, skin diseases, diabetes, and the use of pain-relieving or psychoactive medications. The study was approved by the Swedish Ethical Review Authority (dnr 2020-04207) and written informed consent was obtained from all participants before the start of the experiment. The study was conducted in accordance with the Helsinki Declaration. Participants were seated comfortably in a chair with their knee flexed to ~130°.

EMG recordings

Three self-adhesive recording electrodes (Kendall ECG electrodes, 57x34 mm, Medtronics, USA) were attached to the right tibialis anterior (TA) muscle serving as active, reference, and ground points. EMG recording settings comprised a 1 mV range, 1 kHz low pass filter, 0.3 Hz high pass filter, and 20 kHz sampling rate (LabChart v8.1.16, ADInstruments, Dunedin, New Zealand).

Intradermal electrical stimulation

Two uninsulated tungsten microelectrodes (FHC Inc., Bowdoin, USA) were inserted just below the metatarsophalangeal joint of the right foot, separated by 5 mm, to deliver focal electrical stimulation. The microelectrodes had a tip diameter of 5-10 µm and a shaft of 200 µm. Each stimulus trial consisted of 5 square pulses delivered at a frequency of 200 Hz with a pulse duration of 0.2 ms. These stimuli were generated using a constant current bipolar stimulator (DS8R, Digitimer, Hydeway, UK). In cases where a reflex could not be evoked with a pulse duration of 0.2 ms, a longer pulse duration of 1 ms was used. The interstimulus intervals were varied from trial to trial (at least >6 s) to prevent habituation and/or cognitive suppression of the reflex response. To avoid visual and auditory cues, a partition was placed between the subject and the experimenter, and a silent mouse was used to deliver the stimuli.

Perception and reflex threshold measurements

Participants were instructed to remain relaxed during the recordings, which were performed without baseline EMG activity. Current intensities were slowly increased in increments of 1-3 mA until the first (nonpainful) sensation was reported. This was taken as the detection threshold (DET^{th}). The same procedure was repeated to establish the minimum current required to evoke a painful percept (pain threshold, $PAIN^{th}$) and the NWR threshold (NWR^{th}). Subjects were asked to rate the intensity of pain on a visual analog scale (VAS) ranging from 0 to 10, with 0 representing “no pain” and 10 representing the “worst imaginable pain” (Response meter, ADInstruments, Dunedin, New Zealand). Pain qualities were captured using a short-form McGill Pain Questionnaire (Melzack, 1987) immediately following $PAIN^{th}$ and NWR^{th} measurements (a total of six times).

Reflex analysis

Reflex latencies, Z scores, and pain ratings were analyzed in MATLAB (r2021b, MathWorks Inc, Natick, Massachusetts). Z scores were calculated as the difference between peak amplitude (50-150 ms post-stimulus onset) and mean baseline amplitude (-0.15 to 0 ms stimulus onset), divided by the standard deviation of baseline EMG activity. The minimum current intensity required to evoke a reflex response was taken as the NWR^{th}. Responses with latencies exceeding 150 ms after stimulus onset were excluded from the analysis to avoid startle responses that are known to follow reflex elicitation (Dowman, 1992).

Nerve block

An ischemic nerve block progressively affects large myelinated fibers that signal vibration, followed by small myelinated fibers that signal innocuous cold, and finally, unmyelinated fibers that signal warmth sensations (Laursen et al., 1999; Mackenzie et al., 1975; Mahns & Nagi, 2013; Nagi et al., 2011; Torebjork & Hallin, 1973). To induce the block, an air-filled pressure cuff (Riester Gmbh, Jungigen, Germany) was placed over the right ankle and inflated to 300 mm Hg for up to an hour (Sharma & Salhotra, 2012). The block was applied distal to the TA muscle EMG was recorded from.

In order to track the progression of the nerve block, vibration perception (test for Aβ-fiber function) was tested on the foot sole in three ways: *1.* With a tuning fork (128 Hz, American Diagnostic Corporation, NY, USA) using a two-alternative forced choice detection task (2AFC) where participants reported whether the tuning fork was perceived as "vibration" or "no vibration"; *2.* With a punctate Piezo electric stimulator (probe diameter: 1.3 cm, Dancer Design, UK) where participants rated the intensity of vibration (20 or 200 Hz) on an analog scale ranging from 0 ("no vibrating sensation") to 10 ("highest vibrating intensity"); *3.* Using a 3AFC detection task where participants reported whether vibration at 200, 20, and 0 Hz was perceived as "high", "low" or "no" vibration, respectively. Participants wore earplugs during vibration tests to prevent auditory cues. When participants could no longer distinguish whether the tuning fork was stationary or vibrating, the blockade of Aβ fibers was considered successful. In addition, it was expected that vibratory stimuli would be rated as less intense during the block, and subjects would be unable to discriminate between 20 and 200 Hz frequencies.

To assess Aδ- and C-fiber functions, cold and warm detection thresholds (CDT and WDT) were measured on the foot sole using the method of limits. The thermode probe had dimensions of 30 x 30 mm (TSA-II, Medoc Ltd., Ramat Yishai, Israel) and the rate of temperature change was set at 1°C/s, starting from a neutral temperature of 32°C (Hilz et al., 1999). Each modality was tested four times. In the condition where the vibration sense was blocked while temperature senses remained, perceptual responses at $PAIN^{th}$ and reflex responses at NWR^{th} were tested (at least 3 times).

In the pre-block condition, reaction time measurements were conducted 10 times at the $PAIN^{th}$ stimulus intensity. During this assessment, subjects were asked to press a button as soon as they felt a painful sensation. The inter-stimulus intervals were pseudorandomized (mean: 3.7 s, min: 1.1 s, max: 7.5 s).

During the block, if any pain was reported by the subjects at the $PAIN^{th}$, the reaction time measurements were tested again.

Upon release of the nerve block, vibratory and thermal sensibilities were monitored and upon recovery to pre-block levels (typically within 20-30 min), $PAIN^{th}$ and NWR^{th} were measured again.

Control experiment

To confirm that any effect of the nerve block on pain and reflex responses was due to the blockade of peripheral Aβ inputs rather than central or other factors, in five subjects we ran control experiments with the ischemic cuff applied to the contralateral (left) leg, while pain and reflex responses were still measured from the standard (right) leg.

Statistical analysis

The experiment followed a quantitative, repeated-measurement design in which participants were always tested in three conditions: before (Baseline), during (Block), and after (Recovery) the nerve block. Descriptive statistics and analyses were performed in Prism (9.0.2, GraphPad Software, San Diego, USA). Non-parametrical statistical tests were chosen because of the dataset's medium to small sample sizes, non-normal distribution (as indicated by normality tests), skewed distribution (QQ-plots, skewness, and kurtosis), and/or high standard deviation (SD) in relation to mean values (>50%). Wilcoxon test was used to compare two related groups. Friedman´s test was used to compare multiple related groups and Kruskal-Wallis for multiple non-related groups. Dunn's test was used as a post hoc for multiple comparisons. Spearman's rank was used to investigate correlations with a 95% confidence interval (CI). A p-value of < 0.05 was considered statistically significant. The a-priori sample size estimation was based on a pilot study where we observed an effect size (f) of 0.255. We then used a 1-β error probability (power) of 0.80, and α error probability of 0.05, which gave a total sample size of 27. Post hoc power analysis, based on f(0.27), α(0.05), and a sample size of 25, gave a power of 82.6. Sample size and power calculations were performed in G* Power (open software, v3.1.9.7). Data are shown as median with interquartile range.

Results

Under baseline conditions, NWR was successfully evoked in 22 out of 25 subjects. The current intensity required to reach DET^{th} was the lowest, followed by $PAIN^{th}$, and finally NWR^{th} (Fig. 1A-B). Consequently, all reflex responses occurred in response to a painful stimulus. A total of 198 NWR responses had Z scores ranging from 1 to 61 and occurred at latencies between 65 and 137 ms after stimulus onset (mean latency: 91 ms). Out of these, 80 (40.4%) NWR responses had latencies under 90 ms, a cutoff used in prior studies (e.g. Ruscheweyh et al., 2011; Sandrini et al., 1993; Ydrefors et al., 2020). In our earlier work involving transcutaneous electrical stimulation, dual reflex responses (RII and RIII) were observed in 12% of reflex recordings (Thorell et al., 2023). In the current study using intradermal electrical stimulation, no instances of dual RII-RIII responses were observed (Fig. 1C). When applying a cut-off of 96 ms derived from the analysis of dual reflex responses (Thorell et al., 2023) to this dataset, 137 NWR responses (69.2%) had latencies under 96 ms. Importantly, reflex responses with short and long latencies were equally painful (Fig. 1D). In a subset of participants (n = 7), the reflex could only be evoked using a 1-ms pulse duration. There were no differences in pain ratings nor NWR latencies at $PAIN^{th}$ and NWR^{th} between the 0.2-ms and 1-ms responses, hence the data were combined (Fig. S1A-D).

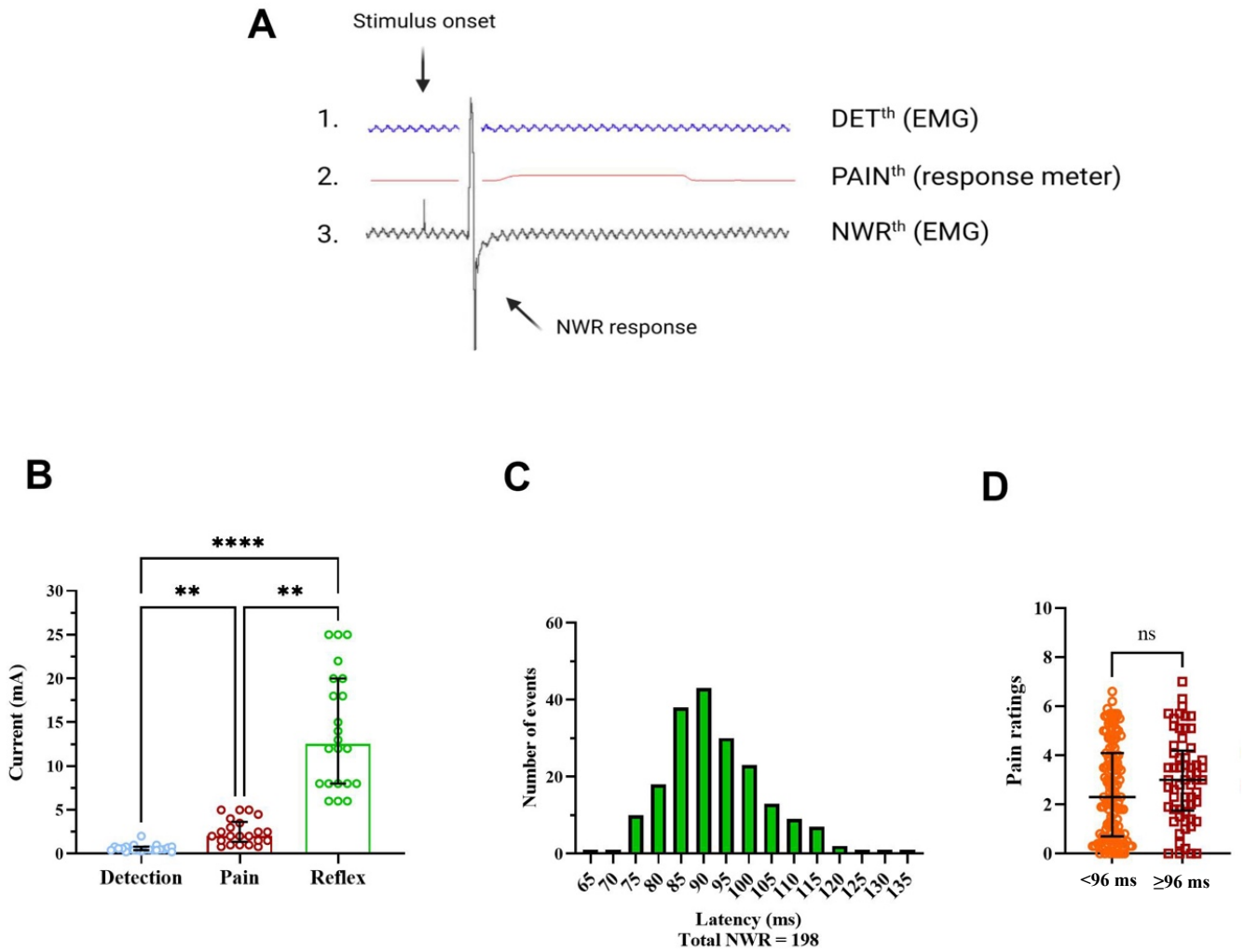

Figure 1. Characterization of pain and reflex responses evoked by intradermal electrical stimulation. A. Raw example traces at thresholds of perception or reflex. The first trace shows the absence of an EMG response at the nonpainful detection threshold (*DETth*). The second trace shows a pain rating at the pain threshold (*PAINth*), although no EMG response was detected. The third trace shows an EMG response at the NWR threshold (*NWRth*). **B. Stimulus intensities for *DETth*, *PAINth*, and *NWRth*.** These were significantly different (*DETth*: 0.5 (0.2) mA, *PAINth*: 2.0 (1.5) mA, *NWRth*: 13.5 (12.0) mA, f(2) = 44.00, $p < 0.0001$, post hoc test: **P = 0.0027; ****P < 0.0001, n = 22, Friedman test). **C. Unimodal distribution of NWR latencies.** All NWR responses (n = 198) were evoked at stimulus intensities perceived as painful by the participants. **D. Pain ratings corresponding to short- and long-latency NWR responses.** Pain ratings did not differ based on NWR latencies. The cut-off at 96 ms was derived from dual reflex responses reported previously (*pain ratings <96 ms*: 2.3 (3.4), *pain ratings ≥96 ms*: 3.0 (2.5), p = 0.108, U = 3581, n = 198, Mann-Whitney test, ns = not significant).

Preferential block of Aβ fibers

Somatosensory tests were performed to gauge the progression of the ischemic nerve block. During baseline and recovery conditions, participants performed with 100% accuracy in the vibration discrimination tasks (2AFC, 3AFC). During the block (for >20 min but <1 hour), subjects could no longer distinguish whether the tuning fork was stationary or vibrating. Further, the vibration intensity ratings declined significantly (Fig. 2A-B), and vibration discrimination was significantly impaired (Fig. 2C).

CDTs were significantly altered (mean difference from baseline = 5.6 °C) during the nerve block (Fig. 2D). Further analysis revealed that among the 22 subjects, 13 had CDT below 23°C, which is considered the lower border of normal values for innocuous cold detection in the foot (Rolke et al., 2006). However, no participant had CDT below 12°C (Magerl et al., 2010), indicating that cooling remained detectable within the nonpainful range during the block. The change in CDT (baseline vs. block) was unrelated to the block duration (Fig. S2A). When comparing pain ratings (at $PAIN^{th}$) between subjects with CDT above or below 23°C, no significant difference was observed (Fig. S2B).

WDTs were not significantly different between baseline and block conditions, or between block and recovery conditions (Fig. 2E). A significant correlation was found between the change in WDT (baseline vs. block) and the duration of the nerve block (Fig. S2C). During the block, none of the subjects reported an abnormal WDT (>45°C) (Rolke et al., 2006), underscoring preserved C-fiber function.

NWR abolished by preferential Aβ-fiber block

During the nerve block, all responses at pre-block NWR^{th} were abolished, suggesting that Aβ afferents have a crucial role in eliciting a functional reflex response (Fig. 2F). Despite further increases in stimulus intensity (up to 2 times the pre-block NWR^{th}) and/or prolonging of the pulse duration (extended to 1 ms), the reflex did not recover during the nerve block.

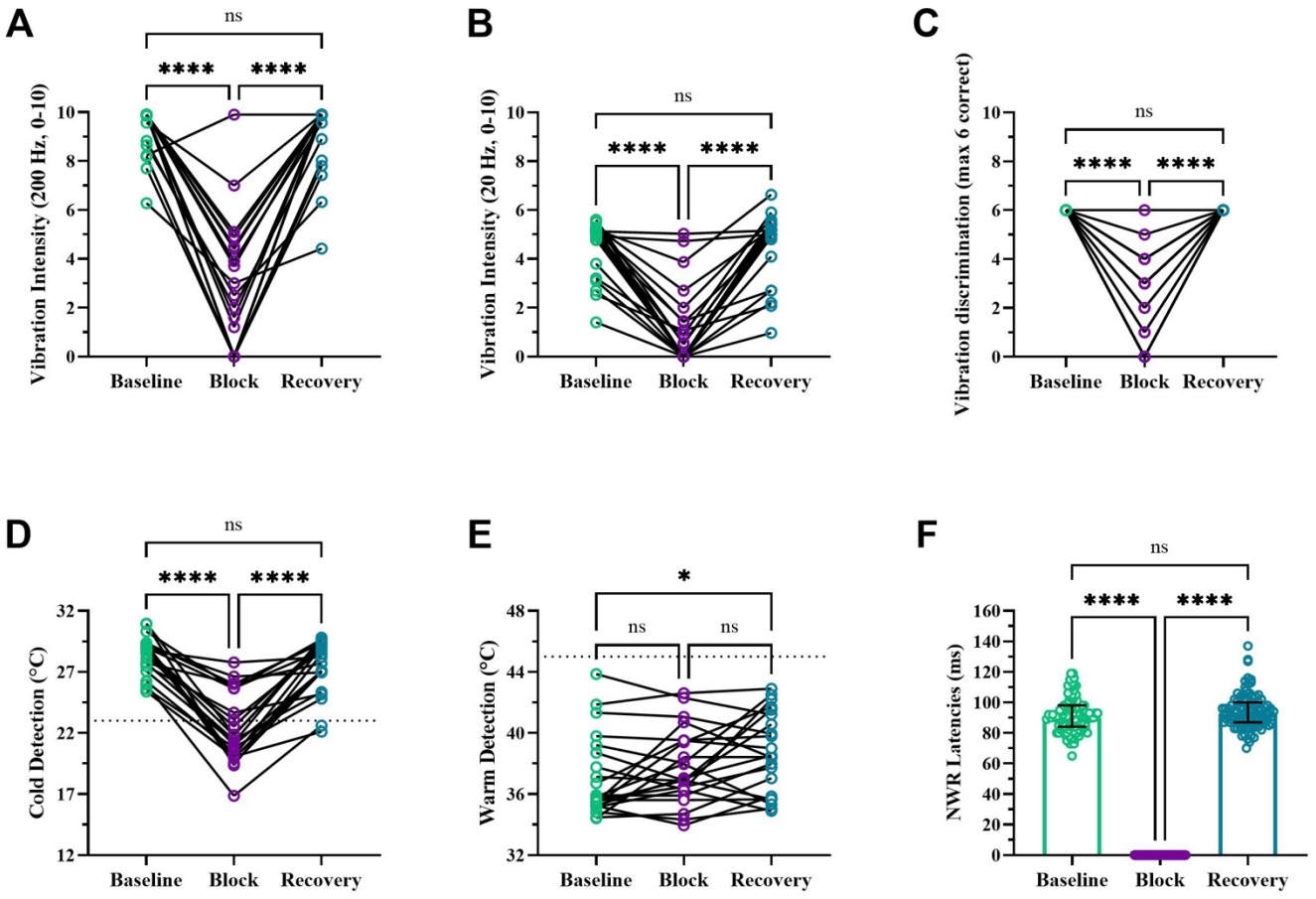

Figure 2. Assessment of vibratory and thermal perception and NWR during nerve block. A-C. Assessment of vibratory function during nerve block. Vibration intensity ratings for 200 Hz (A) and 20 Hz (B) declined significantly during the nerve block (*200 Hz*: baseline 9.9 (1.4), block 1.8 (4.4), recovery 9.9 (1.2), f(2) = 32.38, p < 0.0001; *20 Hz*: baseline 5.1 (1.6), block 0.3 (1.6), recovery 5.1 (1.7), f(2) = 33.77, p < 0.0001, post hoc test: ****P < 0.0001, ns > 0.9999, n = 22, Friedman test). Vibration discrimination (6 trials per condition) was also significantly impaired during the block (*3AFC*: baseline 6.0 (0.0), block 3.0 (3.0), recovery 6.0 (0.0), f(2) = 40, p < 0.0001, post hoc test: ****P < 0.0001, ns > 0.9999, n = 21, Friedman test). **D-E. Assessment of thermal functions during nerve block.** Cold detection thresholds (CDTs) significantly changed during the block (baseline 28.8 (1.9)°C, block 21.6 (5.6)°C, recovery 28.0 (2.8)°C, f(2) = 35.27, p < 0.0001, post hoc test: ****P < 0.0001, ns = 0.395, n = 22, Friedman test). The dotted line at 23°C represents the lower border of normal values for innocuous cold detection, with cold pain emerging below 12°C. Warm detection thresholds (WDTs) remained unchanged during the block but were elevated in the recovery condition compared to baseline (baseline 35.8 (3.6)°C, block 37.0 (3.3)°C, recovery 38.4 (5.5)°C, f(2) = 7.44, p = 0.024, post hoc test: *P = 0.031, ns (baseline vs block) > 0.9999, ns (baseline vs recovery) = 0.150, n = 22, Friedman test). **F. NWR**

abolished during nerve block. The reflex responses were completely abolished during the nerve block (NWR latencies: baseline 90.0 (14.0) ms, block 0.0 (0.0) ms, recovery 92.5 (13.0) ms, $f(2) = 166.4$, $p < 0.0001$, post hoc test: ****$P < 0.0001$, ns = 0.458, n = 198, Kruskal-Wallis test).

Reduced pain during preferential Aβ-fiber block

During the nerve block, pain ratings at the pre-block $PAIN^{th}$ current intensity dropped significantly, resulting in the complete abolition of pain in 14 out of 22 participants (Fig. 3A). Reaction time measurements revealed that any residual pain sensation at the pre-block $PAIN^{th}$ current intensity was significantly delayed (baseline 258.8 ms, block 426.2 ms, n=6), suggesting perception mediated via slower-conducting first-order afferents (Fig. S3A). In four subjects, pain ratings at $PAIN^{th}$ increased during the nerve block, an effect unrelated to the block duration (Fig. S3B). Further, these four subjects were not different from the other participants when comparing reflex latencies, pain ratings at $PAIN^{th}$ or NWR^{th}, vibration sensibility, or temperature thresholds across conditions (Fig. S3C-H).

The most frequently chosen descriptors for characterizing pain quality at $PAIN^{th}$ were “sharp” and “stabbing”. At NWR^{th}, “shooting” and “hot-burning” were also frequently chosen. The proportion of descriptor intensity ranked as moderate and severe increased with increasing stimulus intensity from $PAIN^{th}$ to NWR^{th} (mild-moderate-severe: 74-21-5% *to* 53-39-8%, respectively; Fig. 3C).

During the block, while the NWR was abolished (Fig. 2F), pain ratings at the pre-block NWR^{th} did not differ from baseline levels (Fig. 3B). However, the overall occurrence of descriptors (and their corresponding intensity) reduced by 71% and 66% at $PAIN^{th}$ and NWR^{th}, respectively (Fig. 3D).

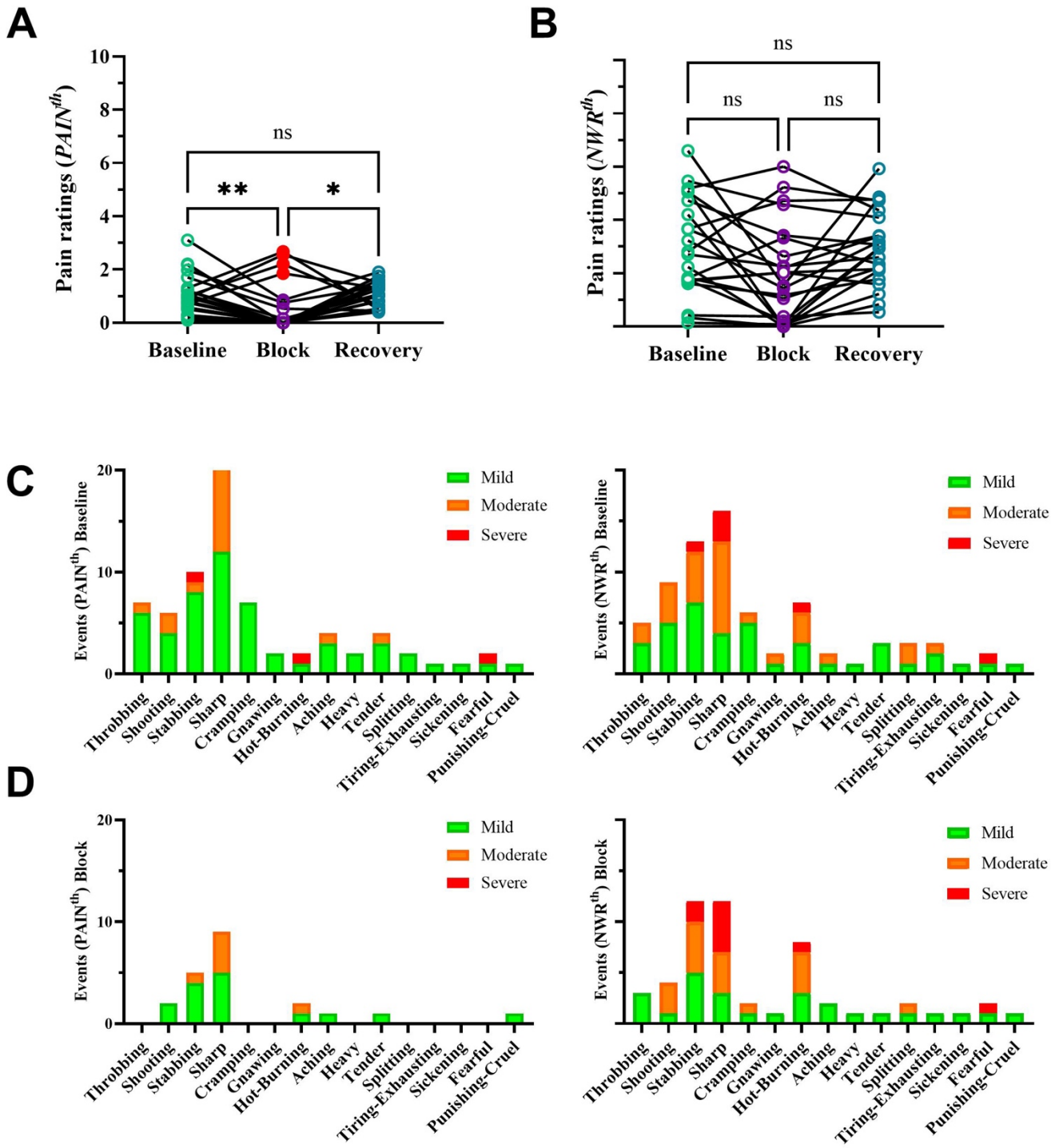

Figure 3. Effect of nerve block on pain intensity and quality. A. Reduction in pain ratings at *PAINth* during nerve block. Pain ratings at the pre-block *PAINth* current intensity were significantly reduced during the block (baseline 0.9 (0.7), block 0.0 (0.7), recovery 1.0 (0.9), f(2) = 11.55, p = 0.003, post hoc test: *P = 0.013, **P = 0.008, ns > 0.999, n = 22, Friedman test). Pain was completely abolished in 14 subjects, greatly reduced in another 4, and increased in the remaining 4 (highlighted in red). **B. No**

effect on pain ratings at *NWRth* during nerve block. Pain ratings at the pre-block *NWRth* current intensity did not significantly change across conditions (baseline 2.8 (3.1), block 1.8 (3.2), recovery 3.0 (2.2), f(2) = 6.181, p = 0.0331, post hoc test: ns (baseline vs block and baseline vs recovery) = 0.071, ns (baseline vs block) > 0.9999, n = 22, Friedman test). **C & D. Reduced occurrence and intensity of descriptors during nerve block.** Pain qualities at the pre-block *PAINth* current intensity are shown on the left, while pain qualities at the pre-block *NWRth* current intensity are shown on the right. On each occasion, participants chose any number of descriptors and ranked their intensity as mild, moderate, or severe. Thus, the maximum number of "events" for each descriptor equals the number of participants (n=22. The y-axis shows how many times a descriptor was chosen, and the x-axis shows the complete list of descriptors from the McGill short-form questionnaire.

In the control experiment, pain ratings at *PAINth* and *NWRth*, as well as NWR latencies, remained unchanged when the nerve block was applied to the contralateral leg (Fig. S4A-C).

Discussion

A preferential Aβ-fiber block significantly reduced pain and led to the complete abolition of NWR responses evoked by intradermal electrical stimulation. These findings suggest the involvement of Aβ fibers in electrical pain perception and NWR signaling. That Aβ afferents contribute to electrical pain perception is consistent with previous findings of reduced mechanical pain perception in patients with selective Aβ deafferentation and normal mechanical pain perception in patients with selective small-fiber deafferentation (diagnosed with hereditary sensory and autonomic neuropathy type V) (Nagi et al., 2019; Ridderström et al., 2020). Among the diverse nociceptor populations, the recently discovered Aβ nociceptors in human skin are particularly well-suited to signal percepts and responses requiring rapid transmission of nociceptive information from the periphery. The activation of Aβ nociceptors using low-current intraneural stimulation produces painful percepts at the same current intensities where Aβ tactile afferents produce nonpainful percepts (Nagi et al., 2019).

In the current study, we found no instances of dual NWR responses. This might be due to the use of focal intradermal stimulation, as the conventional approach to evoke the reflex involves surface electrodes. However, in a previous study using surface electrical stimulation, we found that only 12.4% of the NWR responses had a dual component (Thorell et al., 2023). While a study using both needle and

surface electrodes noted the absence of the early component of the reflex response (RII) during surface stimulation (de Willer, 1977), another study found no difference between the two methods (Meinck et al., 1981). Focal intradermal electrodes, as used in the current study, may stimulate the terminal branches of cutaneous afferents instead of the nerve endings directly, resulting in a less synchronized afferent volley and resembling a more natural stimulus compared to surface electrodes (Shahani & Young, 1971). Differences in the targeted nerves (sural versus tibial) or stimulation paradigms (duration and number of pulses) could be other potential factors for the absence of dual NWR responses in our study.

In the literature, the short-latency component is considered non-nociceptive with the fast-conducting presumed "tactile" inputs thought to serve a role in posture correction or the inhibition of the late reflex response (Ellrich & Treede, 1998; Hugon, 1973; Shahani & Young, 1971). The NWR responses in our data had latencies ranging from 65 to 137 ms, corresponding to a mix of RII and RIII latencies; however, they were equally painful. Based on the current and our previous findings (Thorell et al., 2023), it is evident that the NWR response does not always comprise a double EMG burst and can be just as painful regardless of reflex latency.

Pulse durations used to evoke NWR responses range from 0.1 to 1 ms (Meinck et al., 1985; Sandrini et al., 2005; Tøorring et al., 1981). In the current study, our preference for short-duration electrical pulses (0.2 ms) was to preferentially activate larger-diameter fibers, based on the strength-duration relationship for electric excitation of myelinated axons (Hill, 1936; Szlavik & de Bruin, 1999; Wesselink et al., 1999). Consistent with earlier studies, and our pilot observations, using a single pulse failed to evoke an NWR response, indicating that temporal summation over the course of the stimulus train is required for reflex elicitation (Meinck et al., 1981; Sandrini et al., 2005; Tøorring et al., 1981). Typically, this need for multiple stimuli is overlooked when calculating conduction velocity in the afferent limb, as measurements are routinely made from the onset of the first pulse which fails to evoke an NWR. As NWR responses are typically elicited by trains of pulses separated by 2-5 ms, the conduction velocity of the afferent fibers contributing to the response could be underestimated, leading to bias towards the slower (Aδ) conduction range. Furthermore, the observations that NWR latencies are reduced by 3-4 ms during voluntary EMG activity (Hagbarth, 1960) and that motor neuronal excitability is enhanced for

50-150 ms following low-threshold electrical stimulation (Shahani, 1970) indicate that the excitability of the motor neuronal pool is a key determinant of the timing and amplitude of the NWR.

NWR not only relies on temporal summation driven by high-frequency repeated stimuli, but it also requires stimulus intensities much higher than those needed for pain perception, indicating the need for more extensive spatial convergence to evoke motor responses *vis-à-vis* the sensory perception. In this context, the high impulse rates (up to 300 Hz) produced by Aβ nociceptors (Nagi et al., 2019) in response to noxious stimuli suggests that this class of afferent fiber is ideally suited for detection (and rapid relay) of information about noxious stimuli and to contribute to the generation of NWR. Indeed, the function of pain was a warning system necessitates the rapid transmission of information from the periphery to execute appropriate motor responses and meet behavioral requirements.

During the block, pain ratings at the pre-block $PAIN^{th}$ current intensity were significantly reduced, along with the frequency of pain descriptors and their corresponding intensities. Interestingly, the most frequently chosen pain descriptor "*sharp*" matches the percept evoked by selective activation of single Aβ nociceptors using low-current intraneural stimulation (Nagi et al., 2019). During the block, overall pain ratings at the pre-block NWR^{th} current intensity did not change, but the frequency of pain descriptors and their corresponding intensities were greatly reduced. It may be that the increase in stimulus intensities from $PAIN^{th}$ to NWR^{th} led to the activation of additional afferent types (Aδ and possibly C fibers), which might explain the persistence of pain at the pre-block NWR^{th} current intensity during the block. This shift towards reliance on small-diameter inputs is reflected in the prolonged reaction times for electrical pain at $PAIN^{th}$ during the block (baseline 258.8 ms, block 426.2 ms), which now fall within the same range as reaction times for cold detection – a known Aδ function (Yarnitsky & Ochoa, 1991).

During the block, while pain ratings at pre-block $PAIN^{th}$ were reduced or abolished in most subjects, in four of them, the pain was intensified. One possible explanation for this increased pain could involve a rebalancing of large-fiber inputs, which were diminished during the Aβ-fiber block, thus leading to an unopposed relay of small-fiber inputs, triggering the opening of the 'gate' that is interpreted as pain (Melzack & Wall, 1965; Mendell, 2014). This implies that depending on the individual 'resting state' and the net sum of nociceptive signaling, the outcome can differ across individuals. Other stimulation

techniques, such as transcutaneous electrical stimulation where high- or low-frequency electrical stimulation is applied to the skin, share a similar mix of opposing gating (analgesic) and facilitatory (algesic) actions that can vary from person to person and over time (Johnson et al., 2022; Richardson et al., 1981).

Nociceptive responses can be modulated by another nociceptive stimulus in a phenomenon known as diffuse noxious inhibitory control (DNIC), where 'pain inhibits pain'. This is caused by inhibition in the spinal dorsal horn by nociceptive input from an adjacent part of the body (Le Bars et al., 1992). This phenomenon has been tested in relation to the NWR, resulting in increased NWR thresholds following exposure to noxious cold, heat, and muscular exercise (Willer et al., 1984). To investigate potential DNIC effects, we performed control experiments with the blood pressure cuff, which itself could be considered a nociceptive stimulus, applied to the contralateral leg. However, we found that pain and the NWR persisted ipsilaterally, indicating that DNIC alone was not responsible for the observed changes, but rather the diminution of pain and the complete abolition of NWR were a result of a loss of Aβ inputs when the cuff was applied to the ipsilateral leg.

During the block, CDT was significantly affected (reduced sensitivity). However, no difference was found in pain ratings at pre-block $PAIN^{th}$ between subjects with reduced and normal cold sensitivity during the block. Importantly, during the block, despite a two-fold increase in pre-block NWR^{th} and a five-fold increase in pulse duration, the reflex could not be evoked, even though all subjects could still detect cooling in the nonpainful range (>12°C) mediated by Aδ fibers. Warm sensibility, a function of C fibers, remained unaffected during the block. WDT values were elevated in the recovery condition compared to baseline, which could possibly be a consequence of increased blood flow to the limb upon cuff release, masking the subjects' ability to detect warm temperatures. A longer waiting time for full recovery (>30 min) might have eliminated that difference.

We made several important observations regarding pain and the NWR in our experimental conditions. The NWR consisted of a single response, evoked at stimulus intensities that were always painful, with no NWR responses observed below the pain threshold. The short-latency reflex responses were found to be equally painful as the late reflex responses, challenging the conventional notion of an early non-nociceptive component. During a preferential block of Aβ afferents, pain at the perception threshold

was diminished, and the NWR was abolished. These results indicate the involvement of very fast-conducting afferents in pain perception and NWR signaling, advancing our understanding of nocifensive mechanisms in humans. These findings may also be relevant for understanding the functions of recently discovered human Aβ nociceptors.

Author contributions

O.T., D.A.M., J.O., and S.S.N. developed the experimental protocol with input from J.L. and H.O. O.T. performed the experiments, with assistance from J.O. at the pilot stage. O.T. analyzed the data, with assistance from M.S. O.T., S.S.N., and D.A.M. wrote the manuscript with input from all other authors. All authors have read and approved the manuscript for submission.

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Conflict of interest

The authors declare no conflict of interest.

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